Offline comprehensive liquid chromatography in combination with a Deoxyribonuclease I immobilized enzymatic reactor for selective screening of oligonucleotide mixtures

Piotr Wiktor Álvarez Porebski[1], Frederic Lynen[2]*

[1,2] *Separation Science Group, Department of Organic and Macromolecular Chemistry, Universiteit Gent, Krijgslaan 281 S4-bis, 9000 Gent, Belgium.*

[1] PiotrWiktor.AlvarezPorebski@ugent.be

[2] Frederic.Lynen@UGent.be

Tel: +32 (0)9 264 9606

Fax: +32 (0)9 264 4998

Abstract

The development of a comprehensive ion-pair chromatography-immobilized enzyme reactor x ion-pair chromatography (IPC-IMER x IPC) methodology for the advanced characterization of DNA/RNA oligonucleotides (ON) mixtures has been carried out. More in detail, a DNase I IMER has been coupled to IPC in the post column configuration, followed by the collection of the eluting fractions and reanalysis by IPC. The effect of the mobile phase over the IMER activity was qualitatively evaluated. The methodology proved to generate relevant ON degradation profiles that might be correlated with the ON stability towards nucleases. Moreover, this platform shows potential for its further implementation in selective analysis of ON mixtures and in mapping studies.

Keywords

Oligonucleotides, immobilized enzyme reactor (IMER), ion-pair chromatography, Deoxyribonuclease I (DNase I), comprehensive liquid chromatography.

1. Introduction

In the last decades, the usage of oligonucleotides (ONs) for the antisense technology has significantly increased. These molecules are essentially unmodified or chemically modified single-stranded DNA or RNA entities. In general, they are relatively short molecules consisting in average of 13-25 nucleotides [1]. They are widely used to modulate the gene expression in various fields including research, biotechnology and therapeutic applications [2]. Basically, antisense oligonucleotides hybridize specifically to a complementary DNA, pre-mRNA or mRNA via Watson-Crick base pairing [3], and in this way, they block the expression of a target gene [1, 4, 5]. Unmodified DNA and RNA oligonucleotides are inherently unstable both in serum and in cells as they are rapidly degraded by exonucleases and endonucleases [1-4, 6]. Several modifications have been implemented to increase the nuclease resistance of ONs including modifications of the sugar moiety, the nitrogen base and of the phosphate group [7]. Although these alterations significantly reduce their predisposition to nuclease degradation, they do not completely inhibit it. As additionally, many of such modifications of the ON backbone also reduce the corresponding hybridization activity necessary for the therapeutic action, an adequate balance between nuclease resistivity and activity needs to be found [1, 8].

The main synthetic impurities and degradation products encountered in these molecules are very closely related to each other and include mainly adduct sequences (n+1, n+2…) or sequence deletions (n-1, n-2…). In addition, other impurities resulting from oxidation, depurination and other bases are also included in a family of closely related impurities that increase the challenges in the resolution of the

main product. Furthermore, the number of impurities can drastically increase when dealing with double stranded interfering RNA (iRNA) as each strand carries its own set of impurities [2, 3].

Due to the vast number of structural and chemical variations available for ONs, conventional 1D based separation techniques are too limited for adequate mapping of all impurities and degradants which can be generated [9-11]. Therefore, the development of comprehensive 2D separation methods capable to resolve this increasing sample complexity imposes itself. The approaches developed thus far for the analysis of ONs include a comprehensive liquid chromatography (LC x LC) method using hydrophilic interaction liquid chromatography x ion-pair chromatography (HILIC x IPC) for ONs up to 10 bases in length [12], and a comprehensive liquid chromatography x capillary gel electrophoresis (LC x CGE) method based on ion-pair chromatography (IPC) and ion exchange chromatography (IEC) as LC techniques in combination with CGE using entangled polymer solutions [13]. This latter LC x CGE platform, proved to deliver increased peak capacities compared to the 1D separation techniques for the separation of ONs of therapeutic sizes.

The combination of separation techniques with immobilized enzyme reactors (IMERs) leads to improved characterization of complex mixtures by allowing prior isolation of the ONs prone to the enzymatic degradation. Recently, a methodology combining a DNase I IMER coupled in the pre-column configuration to LC was proposed for ON stability assessments [14]. Pure ON samples were thereby subjected to analysis in order to generate a characteristic degradation profile. However, in the presence of samples containing multiple ONs in combination with other molecules, a characteristic ON degradation profile cannot be achieved independently for each entity.

By merging the concept of comprehensive 2D separations with the use of in-line enzymatic reactors, the development of a methodology that is more selective, which depicts more peak capacity and which allows the generation of specific ON stability information becomes possible. Similar platforms (LC-IMER

x LC) have been implemented before for the advanced characterization and/or sample preparation of mixtures of peptides and proteins [15-21].

In this study, we present the highlights from the development of a comprehensive LC-IMER x LC methodology based on ion pair chromatography for separations and applying reactors with immobilized Deoxyribonuclease I. It is illustrated that this tool can be used for comparative assessment of the stability of particular ONs in mixtures of DNA and RNA. To the best of our knowledge, the development of a comprehensive LC-(DNase I) IMER x LC platform applied to ONs had not been developed yet, and could be of relevance as novel tool in the development of antisense ON therapies.

2. Experimental

2.1 Chemicals

Triethylamine (TEA), acetic acid, hydrochloric acid, Tris, , $CaCl_2$, $MgCl_2$, glycine, (3-Glycidyloxypropyl) trimethoxysilane (GPTMS) and Deoxyribonuclease I (DNase I) from bovine pancreas (≥400 Kunitz units/mg of protein) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (ACN) HPLC grade from Fischer Scientific (Loughborough, U.K.) and Milli-Q water (Millipore, Milford, MA) were used. All the mobile phases were filtered through 0.22 μm nylon membrane filters (Grace Davison Discovery Sciences, Lokeren, Belgium). The oligonucleotides: (DNA 12) 12-*mer DNA* ON (5'-GCA-CAC-CGT-CAG-3'), (AC 15) 15-*mer DNA* ON (5'-AAA-CCC-AAA-CCC-AAA-3'), (DNA 20) 20-*mer DNA* ON (5'-AGC-GAT-AAG-ATT-CAT-ATA-TC-3'), 30-*mer DNA ON* of Homo-deoxythymidine (T30), (R41) 41-*mer DNA* ON (5'- GTT-GGA-TTA-AAC-AAC-CGT-TCC-CGT-CTC-TAT-CAG-CTT-AGT-GT-3') and the 2'-OMe *RNA* 12-*mer* ON (5'-GCA-CAC-CGU-CAG-3') were purchased from Eurogentec (Liege, Belgium). The lyophilized ONs were dissolved in an appropriate volume of Milli-Q water in order to obtain 100 μM stock solutions.

2.2 Instrumentation

An Agilent 1200 series HPLC capillary system composed of a binary pump and a single wavelength detector was employed for the delivery of the chromatographic mobile phases, while the enzymatic reaction buffer was delivered to the reactor by a HP 1050 quaternary pump (Agilent Technologies, Waldbronn, Germany). A CTO-20AC prominence HPLC column oven (Shimadzu, Kyoto, Japan) was used for controlling the temperature of the IMER. The collection of the fractions was carried out using a micro fraction collector Agilent 1200 series. The detector wavelength and sampling frequency were set at 260 nm and 10 Hz, respectively. The contour plots were constructed using MATLAB (Natick, MA, USA).

2.3 Preparation of the IMER

An IMER manufactured and characterized in a previous study has been used in the development of this comprehensive platform [14]. In brief, DNase I was immobilized on epoxy activated silica particles (Nucleosil 5 μm, 4000 Angstrom pore size from Macherey-Nagel, Duren, Germany) using GPTMS as activator, while glycine was used for deactivating the unreacted epoxy groups. The DNase I immobilized silica particles (4.9 ± 0.1 mg of DNase I /g of silica) presented a specific activity of 361.8 ± 23 pmol T30/min·g. This activity was determined using a T30 ON as a substrate in a medium consisting of 50 mM Tris buffer pH 7.5, 5 mM $CaCl_2$ and 5 mM $MgCl_2$ at 37 °C. Afterwards, the enzyme-silica particles were slurry packed into 150 mm x 2.1 mm I.D. stainless steel columns at a constant pressure of 400 bar.

2.4 Comprehensive IPC-IMER-IPC setup

2.4.1 First dimension, IPC-IMER

The employed instrumental setup is depicted in Figure 1A. This fully automated procedure can be subdivided into three main events: the chromatographic separation of the ON mixture (i), reaction of the analytes coming from the chromatographic effluent in the IMER (ii), and the collection of fractions eluting from the IMER (iii).

(i) IPC was employed as separation technique in the first dimension. For this, a 100 mm x 1 mm I.D. x 3.5 µm Xbridge C18 column (Waters, Zellik, Belgium) was used. The column temperature was set at 60 °C. The mobile phase was composed of 100 mM TEA (pH 5.5 adjusted with acetic acid) (A) and ACN (B). A flow rate of 20 µL/min was used with the following gradient program: 0–300 min, 0–19.2 %B. Pump 1, which delivered the chromatographic separation conditions, was directly connected to the HP 1200 auto-injector (10 µL of injection volume) followed by the chromatographic column.

(ii) Consequently, the effluent of the chromatographic column was mixed with a concentrated reaction buffer solution (pH 7.5; 1.25 M Tris, 55 mM $CaCl_2$ and 55 mM $MgCl_2$) delivered by Pump 2 with the aid of a T-piece followed by a 2 µL mixing loop (0.127 mm I.D.). The flow rate delivered by Pump 2 was set to 2 µL/min in order to approximate a 1/10 dilution of the reaction buffer with the chromatographic mobile phase. The resulting concentration of the enzyme activating cations (Ca^{2+} and Mg^{2+}) and pH in the mixture mobile phase-buffer were the same as to the one tested in the activity of the IMER (Section 2.3). Subsequently, this effluent was percolated at a total flow rate of 22 µL/min through the IMER kept at 37 °C. The system's and reactor's dead volumes were determined by injecting a 1 mM caffeine

solution. This allowed the estimation of the residence time that the analytes experienced in the IMER (~20 min).

(iii) Thereafter, the effluent from the IMER was connected to a UV detector followed by a fraction collector. Finally, fractions of this first dimension were collected every 120 s into 100 µL vial inserts.

2.4.2 Second dimension, IPC

The fractions collected from the first dimension (Section 2.4.1) were injected (35 µL injection volume) into a conventional HPLC system setup employing IPC as separation technique (Figure 1B). The separation was carried out in a 50 mm × 4.6 mm I.D. × 3 µm XBridge C18 column (Waters, Zellik, Belgium). The column temperature was maintained at 60 °C. The mobile phase consisted of 100 mM TEA (pH 5.5 adjusted with acetic acid) (A) and ACN (B). A flow rate of 0.5 mL/min was used with the following gradient program: 0-50 min, 0-16 %B.

3. Results and discussion

Despite the unavoidable obtained reduction in enzymatic activity after immobilization on a solid support, the obtained gain in mechanical and chemical resistance of the enzyme is one of the most important features of this process when comparing to free enzymes in solution. The employed DNase I IMER proved to have an exceptional life time with intrinsic activities capable to generate degradation profiles of DNA based ONs in a reproducible way when combined with liquid chromatography in the pre-column configuration [14]. In the present study, concept of employing the Deoxyribonuclease based IMERs in the post-column configuration is introduced and evaluated. For this, the preservation or suppression of the enzymatic activity was the key evaluated aspect.

In a previous study, the hyphenation of both ion-pair chromatography (IPC) and ion exchange chromatography (IEC) in an IMER-LC setup proved to be suitable [14]. Those LC techniques are also among the preferred ones when seeking for robust high performance separations of ONs, and were therefore evaluated as candidates for the LC-IMER setup. The harsh properties of the mobile phases of those LC techniques (IPC: TEA 100 mM pH 5.5; IEC: ~1.2 M NaCl pH >10 [14]) are in first instance inadequate for the activity of DNase I. Nevertheless, when mixed or diluted with a buffer of satisfactory ionic strength and buffer capacity, suitable conditions for the enzymatic activity may be obtained. In an online LC-IMER setup, the establishment of appropriate conditions for the enzymatic activity may be attained by two strategies: (i) dilution of the mobile phase in the activity buffer, (ii) addition of concentrated activity buffer to the mobile phase. Both possibilities were explored by incubating immobilized DNase I particles in individual vials with the appropriated solvent compositions. The DNase I silica particles were thereby mixed with LC mobile phase compositions which were either diluted or contained an addition of a concentrated activity buffer, this in an effort to simulate conditions that might be obtained the online approach. These test mixtures were stirred for 60 min with the 41-*mer* *DNA* ON and afterwards analyzed by IPC. Figure 2 depicts the chromatograms in which the presence or loss in the enzymatic activity can be visually appreciated. A dilution 1/10 of the IPC mobile phase gradient at the end of the gradient (82% TEA 100 mM pH 5.5, 18% ACN) in the activity buffer (50 mM Tris buffer pH 7.5, 5 mM $CaCl_2$ and 5 mM $MgCl_2$) results in approximately 1.8% of ACN in in the final mixture, while the concentration of the cations Ca^{2+} and Mg^{2+} is not significantly changed to diminish the enzymatic activity. The chromatogram corresponding to this latter mixture is depicted in Figure 2A, in which it can be noticed that the enzymatic activity has not been lost, as the resulting chromatogram (solid-red) presents the same degradation peaks as the chromatogram (dashed-green) corresponding to a sample in which the DNase I silica particles were only incubated in the activity buffer. An overlay with a blank chromatogram (dashed-blue) corresponding to the ON incubated with deactivated silica

particles without enzyme is also displayed, in which the peak eluting at 32 minutes corresponds to the unreacted ON. The pH of the resulting IPC mobile phase-activity buffer was measured as 7.3, which proves to be adequate for the enzymatic activity. In a similar way, a small addition of a concentrated activity buffer solution to the IPC mobile phase (section 2.4.1) also allowed obtaining adequate, yet somewhat less performant conditions for the enzymatic activity (Figure 2B). It can therefore be visually appreciated that the ON main peak has not completely reacted as was the case in the chromatogram presented in Figure 2A. This effect can be mainly attributed to the higher presence of acetonitrile and due to the increased ionic strength of the resulting mixture when compared to a dilution process of the IPC mobile phase. In Figure 2C, the effect on the enzymatic activity of the 1/10 dilution of an IEC mobile phase at the end of the gradient (80%: 20/80 ACN/water 1.25 M NaCl (pH 11.5); 20%: 20/80 ACN/water (pH 11.5)) [13] with the activity buffer is presented. It can be noticed that the increased ionic strength of the medium also does not allow for a complete degradation of the ON. Moreover, when performing an addition of activity buffer to the IEC mobile phase, no enzymatic activity could be appreciated (chromatogram not shown), making this approach unsuitable. An accurate estimation of the enzymatic activity at other dilution factors and mobile phase compositions is possible. Nevertheless, its calculation falls out of the main scope of this study at this stage, where the main message is to point out the possibility of coupling DNase I based reactors between both IPC dimensions of an off-line comprehensive set-up.

From a practical point of view, the addition of activity buffer to the LC mobile phase presents several advantages. First and most importantly, there is minimal dilution of the LC effluent which could result in poor detectability in the second dimension. Second, in order to allow degradation of the ON in the IMER, long residence times need to be established, meaning that the cumulative flow rate from combining the LC mobile phase in the first dimension plus the activity buffer needs to be as low as possible. In the described setup (section 2.4.1), a flow rate of 20 µL/min inevitable requires the usage of

a column with a narrow I.D. of 1 mm, this in order to operate the column close (yet somewhat below) to the optimal linear velocity to obtain satisfactory chromatographic separation performance. However, under these conditions the separation of a mixture of 6 ONs requires 240 min (Figure 3A). Therefore, the implementation of even lower flow rates cannot be considered convenient. On the other hand a 1/10 dilution of the complete effluent of this mobile phase would result in total flow rate through the IMER of 200 µL/min, for which we encountered insufficient reaction of the ONs using this IMER [14]. This latter approach may be improved by the usage of more active IMERs. Another strategy for implementing the dilution would consist in splitting the LC mobile phase before mixture with the buffer. However, that approach is the less preferable as it complicates the experimental setup in addition to drastically reducing the detectability of the ONs in the second dimension.

Figure 3B presents the contour plot of an IPC-IMER x IPC comprehensive analysis of a mixture of 6 ONs. It also confirms that the IMER activity is not lost after performing the online LC-IMER setup with addition of activity buffer to the IPC mobile phase. It can also be observed in Figure 3C that the degradation profile of the 41-*mer DNA* ON is similar to the one obtained in batch analysis (Figure 2B). Moreover, it can be noticed that the degradation profiles are specific for each ON, proving the applicability of this approach for an improved characterization of mixtures of ONs. In Figure 4, an overlay of the IPC-IMER x IPC plot with a blank plot generated using a deactivated IMER without enzyme is presented, whereby it can be observed that certain ONs degrade more than others. These differences are attributed to the nucleotide composition, purine pyrimidine ratio and to the ability of an ON to hybridize between itself. DNase I cleaves preferentially double strands [22], this effect can be particularly visualized for the R41 ON, which due to its length and random nucleotide composition is more likely to form double strands between itself when compared to the shorter ONs (DNA12, AC15, and DNA20). Additionally, the flexibility of the DNA strand also influences the degree of cleavage imposed by the DNase I [23]. DNA regions rich in A and T nucleotides are less flexible [24], explaining the lower cleavage

observed for the T30 ON. The selectivity of the enzyme is also illustrated through its degradation of the DNA molecules while imposing no degradation on the RNA bases ONs. The molecules used here offer a limited number of examples to illustrate the high selectivity provided by IMERs in such a comprehensive platform.

4. Conclusion

A comprehensive IPC-IMER x IPC platform for an improved characterization of mixtures of ONs has been developed. A DNase I IMER was successfully coupled online with IPC in the post column configuration. This proves the possibility for the implementation of other orthogonal LC techniques such as IEC in the first dimension and/or second dimension for instance. The hyphenation of MS detection with a suitable LC technique in the second dimension is realistic and would provide additional valuable information on the cleaved ON fragments. Moreover, the usage of an IMER combined with LC provides reproducible reactions over longer periods of time, as demonstrated before [14]. The possibility to couple IMERs with nucleases which are specific or not to the sugar moiety in addition to their cleavage mechanism (endonuclease, exonuclease) may also be explored. This last approach may be performed either by the simultaneous immobilization of various nucleases to a support or by the interconnection of IMER subunits as many nucleases show similar activation conditions. Finally, the introduction of an enzymatic reaction in a comprehensive type of platform expands its selective power, while providing unique data regarding the stability of mixtures of ONs towards nucleases. The approach offer potential applications for the mapping, characterization and stability assessments of ONs.

Acknowledgments

P.A.P. gratefully acknowledges the National Council of Science and Technology of Mexico (CONACyT) and the Special Research Fund (BOF) of Ghent University for supporting this project. In addition, the authors also thank Prof. Annemieke Madder from the Organic and Biomimetic Chemistry Research Group of Ghent University for proving a number of oligonucleotide samples.

References

[1] N. Dias, C.A. Stein, Antisense oligonucleotides: Basic concepts and mechanisms, Mol Cancer Ther, 1 (2002) 347-355.
[2] C.F. Bennett, E.E. Swayze, RNA Targeting Therapeutics: Molecular Mechanisms of Antisense Oligonucleotides as a Therapeutic Platform, Annual Review of Pharmacology and Toxicology, 50 (2010) 259-293.
[3] R. Kole, A.R. Krainer, S. Altman, RNA therapeutics: beyond RNA interference and antisense oligonucleotides, Nat Rev Drug Discov, 11 (2012) 125-140.
[4] K.J. Myers, N.M. Dean, Sensible use of antisense: how to use oligonucleotides as research tools, Trends Pharmacol Sci, 21 (2000) 19-23.
[5] S. Agrawal, Antisense oligonucleotides: Towards clinical trials, Trends Biotechnol, 14 (1996) 376-387.
[6] M.Y. Skoblov, Prospects of antisense therapy technologies, Molecular Biology, 43 (2009) 917-929.
[7] S. Verma, F. Eckstein, Modified oligonucleotides: Synthesis and strategy for users, Annu Rev Biochem, 67 (1998) 99-134.
[8] S.M.A. Rahman, T. Baba, T. Kodama, M.A. Islam, S. Obika, Hybridizing ability and nuclease resistance profile of backbone modified cationic phosphorothioate oligonucleotides, Bioorgan Med Chem, 20 (2012) 4098-4102.
[9] M. Gilar, K.J. Fountain, Y. Budman, U.D. Neue, K.R. Yardley, P.D. Rainville, R.J. Russell, J.C. Gebler, Ion-pair reversed-phase high-performance liquid chromatography analysis of oligonucleotides: Retention prediction, J Chromatogr A, 958 (2002) 167-182.
[10] A.C. McGinnis, B.Y. Chen, M.G. Bartlett, Chromatographic methods for the determination of therapeutic oligonucleotides, J Chromatogr B, 883 (2012) 76-94.
[11] S. Studzinska, B. Buszewski, Evaluation of ultrahigh-performance liquid chromatography columns for the analysis of unmodified and antisense oligonucleotides, Anal Bioanal Chem, 406 (2014) 7127-7136.
[12] Q. Li, F. Lynen, J. Wang, H.L. Li, G.W. Xu, P. Sandra, Comprehensive hydrophilic interaction and ion-pair reversed-phase liquid chromatography for analysis of di- to deca-oligonucleotides, J Chromatogr A, 1255 (2012) 237-243.
[13] P.A. Porebski, F. Lynen, Combining liquid chromatography with multiplexed capillary gel electrophoresis for offline comprehensive analysis of complex oligonucleotide samples, J Chromatogr A, 1336 (2014) 87-93.
[14] P.W.A. Porebski, E. Gyssels, A. Madder, F. Lynen, Hyphenation of a Deoxyribonuclease I immobilized enzyme reactor with liquid chromatography for the online stability evaluation of oligonucleotides, J Chromatogr A, 1422 (2015) 18-26.

[15] Q. Wu, H.M. Yuan, L.H. Zhang, Y.K. Zhang, Recent advances on multidimensional liquid chromatography-mass spectrometry for proteomics: From qualitative to quantitative analysis-A review, Anal Chim Acta, 731 (2012) 1-10.
[16] C. Temporini, E. Perani, F. Mancini, M. Bartolini, E. Calleri, D. Lubda, G. Felix, V. Andrisano, G. Massolini, Optimization of a trypsin-bioreactor coupled with high-performance liquid chromatography-electrospray ionization tandem mass spectrometry for quality control of biotechnological drugs, J Chromatogr A, 1120 (2006) 121-131.
[17] Y. He, W.W. Zhong, E.S. Yeung, Multiplexed on-column protein digestion and capillary electrophoresis for high-throughput comprehensive peptide mapping, J Chromatogr B, 782 (2002) 331-341.
[18] E.C.A. Stigter, G.J. de Jong, W.P. van Bennekom, Pepsin immobilized in dextran-modified fused-silica capillaries for on-line protein digestion and peptide mapping, Anal Chim Acta, 619 (2008) 231-238.
[19] H.M. Yuan, Y. Zhou, S.M. Xia, L.H. Zhang, X.D. Zhang, Q. Wu, Z. Liang, Y.K. Zhang, Integrated Platform for Proteome Profiling with Combination of Microreversed Phase Based Protein and Peptide Separation via Online Solvent Exchange and Protein Digestion, Analytical Chemistry, 84 (2012) 5124-5132.
[20] J.F. Ma, J.X. Liu, L.L. Sun, L. Gao, Z. Liang, L.H. Zhang, Y.K. Zhang, Online Integration of Multiple Sample Pretreatment Steps Involving Denaturation, Reduction, and Digestion with Microflow Reversed-Phase Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry for High-Throughput Proteome Profiling, Analytical Chemistry, 81 (2009) 6534-6540.
[21] A.M. Girelli, E. Mattei, Application of immobilized enzyme reactor in on-line high performance liquid chromatography: A review, J Chromatogr B, 819 (2005) 3-16.
[22] E. Junowicz, J.H. Spencer, Studies on Bovine Pancreatic Deoxyribonuclease-A .2. Effect of Different Bivalent Metals on Specificity of Degradation of DNA, Biochim Biophys Acta, 312 (1973) 85-102.
[23] B. Heddi, J. Abi-Ghanem, M. Lavigne, B. Hartmann, Sequence-Dependent DNA Flexibility Mediates DNase I Cleavage, J Mol Biol, 395 (2010) 123-133.
[24] A.J. Doherty, A.F. Worrall, B.A. Connolly, The Roles of Arginine-41 and Tyrosine-76 in the Coupling of DNA Recognition to Phosphodiester Bond-Cleavage by Dnase-I - a Study Using Site-Directed Mutagenesis, J Mol Biol, 251 (1995) 366-377.

Figure 1. Schematic representation of the LC-IMER-LC setup. A) First dimension LC-IMER, B) second dimension LC. In the first dimension, an ON mixture is separated by IPC and the effluent is subjected to a reaction in the IMER. The IMER effluent is thereafter connected to a fraction collector. The fractions collected from the first dimension are subsequently directly injected into the second dimension and analyzed.

Figure 2. IPC chromatograms of an R41 ON representing the effect of mixing IPC and IEC LC mobile phases with the activity buffer over the immobilized DNase I activity. The blue small dotted line chromatogram corresponds to the R41 ON incubated with blank silica particles. The green dotted line chromatogram corresponds to the R41 ON incubated with DNase I silica particles in the activity buffer. The solid red line chromatogram corresponds to the ONs incubated with DNase I silica particles in the respective mixture with the mobile phase activity buffer. The encircled region points out the degradation degree of the ON which can be visually estimated as a decrease of its peak height compared to the blank. A) 1/10 dilution of an IPC mobile phase (MP) in activity buffer (resulting in ~1.8% of B in activity buffer). B) Addition of a concentrated activity buffer solution to the IPC MP (resulting in ~18% of B in activity buffer). C) 1/10 dilution IEC mobile phase (MP) in activity buffer (resulting in ~8.0% of B in activity buffer). (For details refer to sections 2.4 and 3).

Figure 3. A) IPC chromatogram of a mixture of 6 ONs. B) IPC-IMER x IPC contour plot. C) IPC chromatogram of a fraction corresponding to the elution of the R41 ON. For conditions refer to section 2.4.

Figure 4. 3D plot overlay of an IPC-IMER x IPC and IPC-(blank IMER) x IPC analysis of a mixture of 6 ONs. For conditions refer to section 2.4.

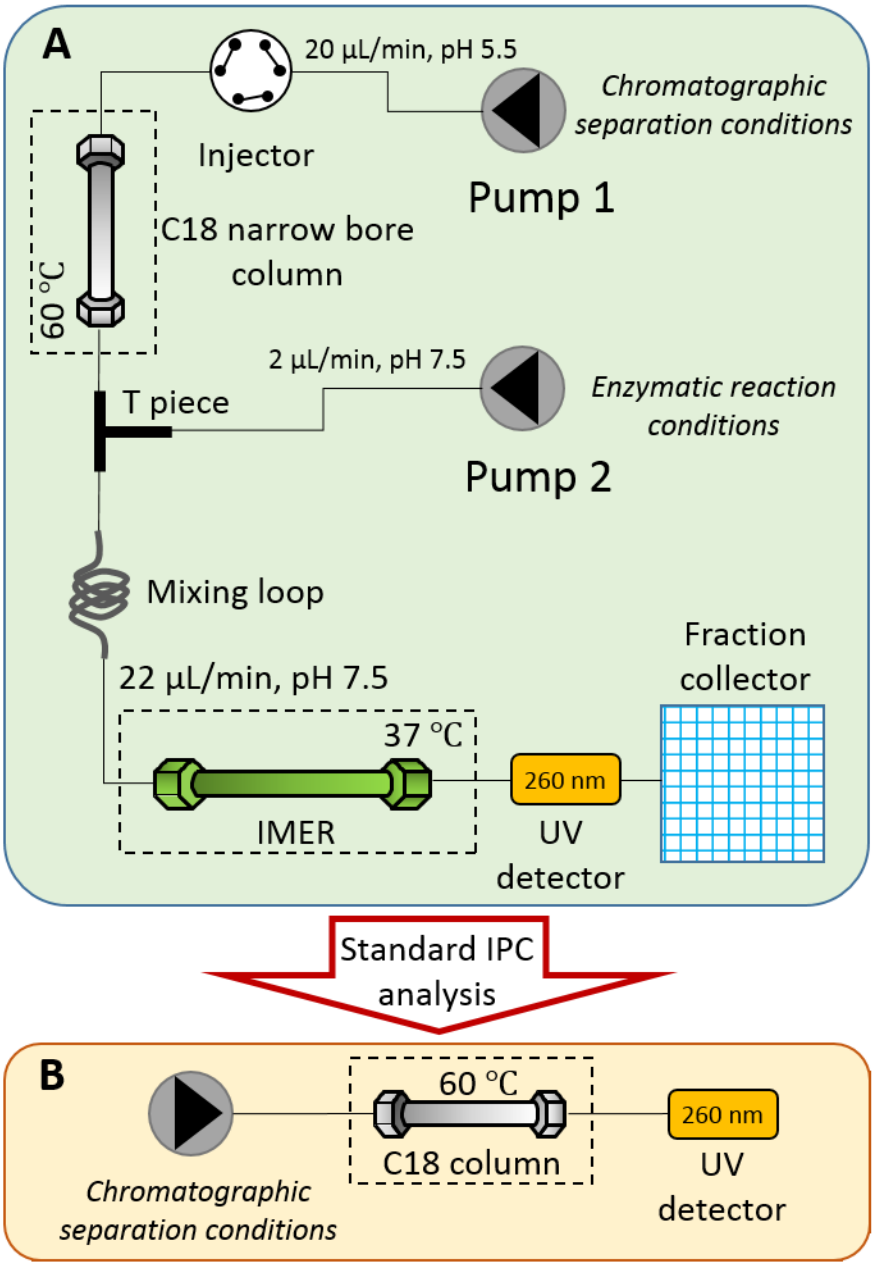


Figure 1. Schematic representation of the LC-IMER-LC setup. A) First dimension LC-IMER, B) second dimension LC.

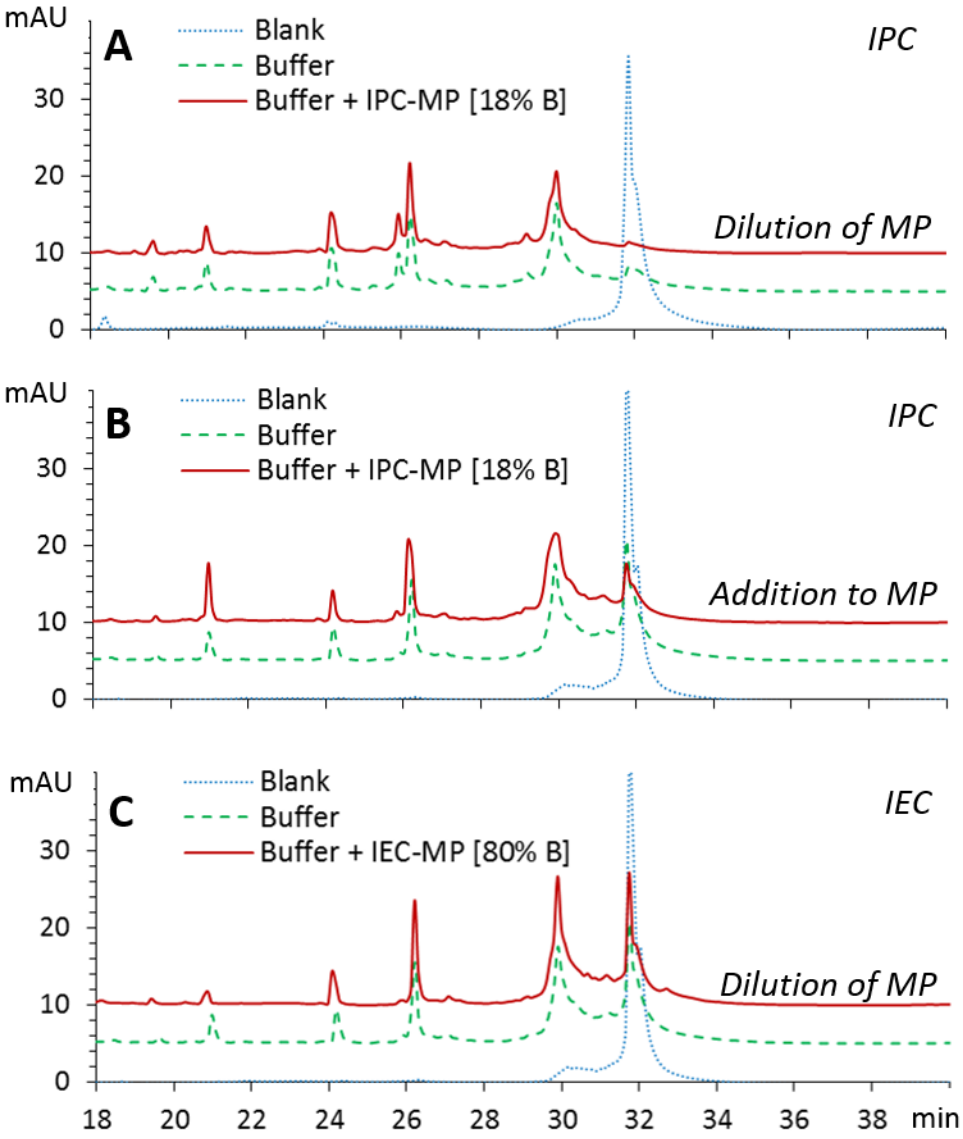


Figure 2. IPC chromatograms of an R41 ON representing the effect of mixing IPC and IEC LC mobile phases with the activity buffer over the immobilized DNase I activity. The blue small dotted line chromatogram corresponds to the R41 ON incubated with blank silica particles. Green dotted line chromatogram corresponds to the R41 ON incubated with DNase I silica particles in the activity buffer. The solid red line chromatogram corresponds to the ONs incubated with DNase I silica particles in the respective mixture of mobile phase activity buffer. A) 1/10 dilution of an IPC mobile phase (MP) in activity buffer. B) Addition of a concentrated activity buffer solution to the IPC MP. C) 1/10 dilution IEC mobile phase (MP) in activity buffer. (For details refer to sections 2.4 and 3).

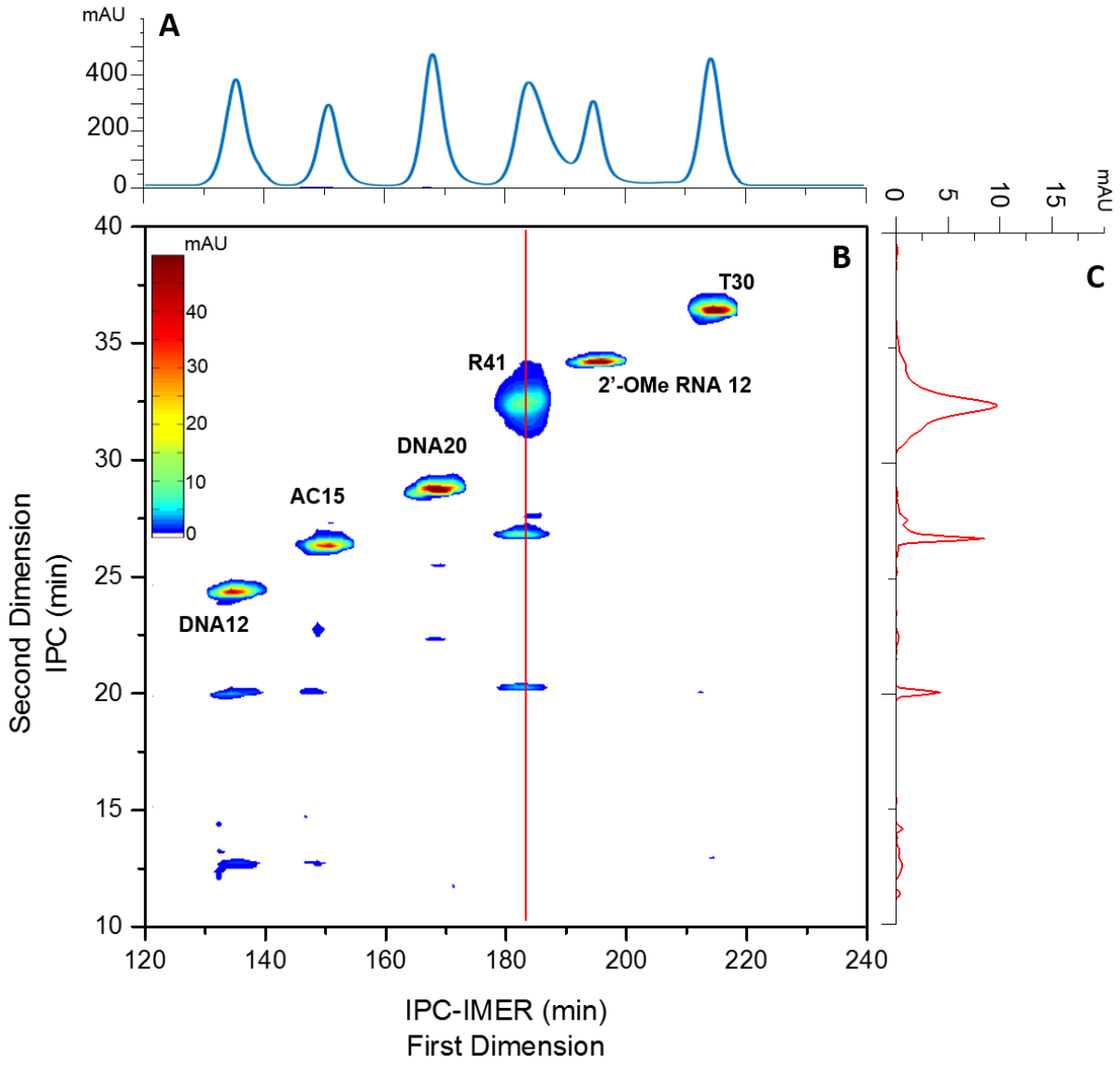
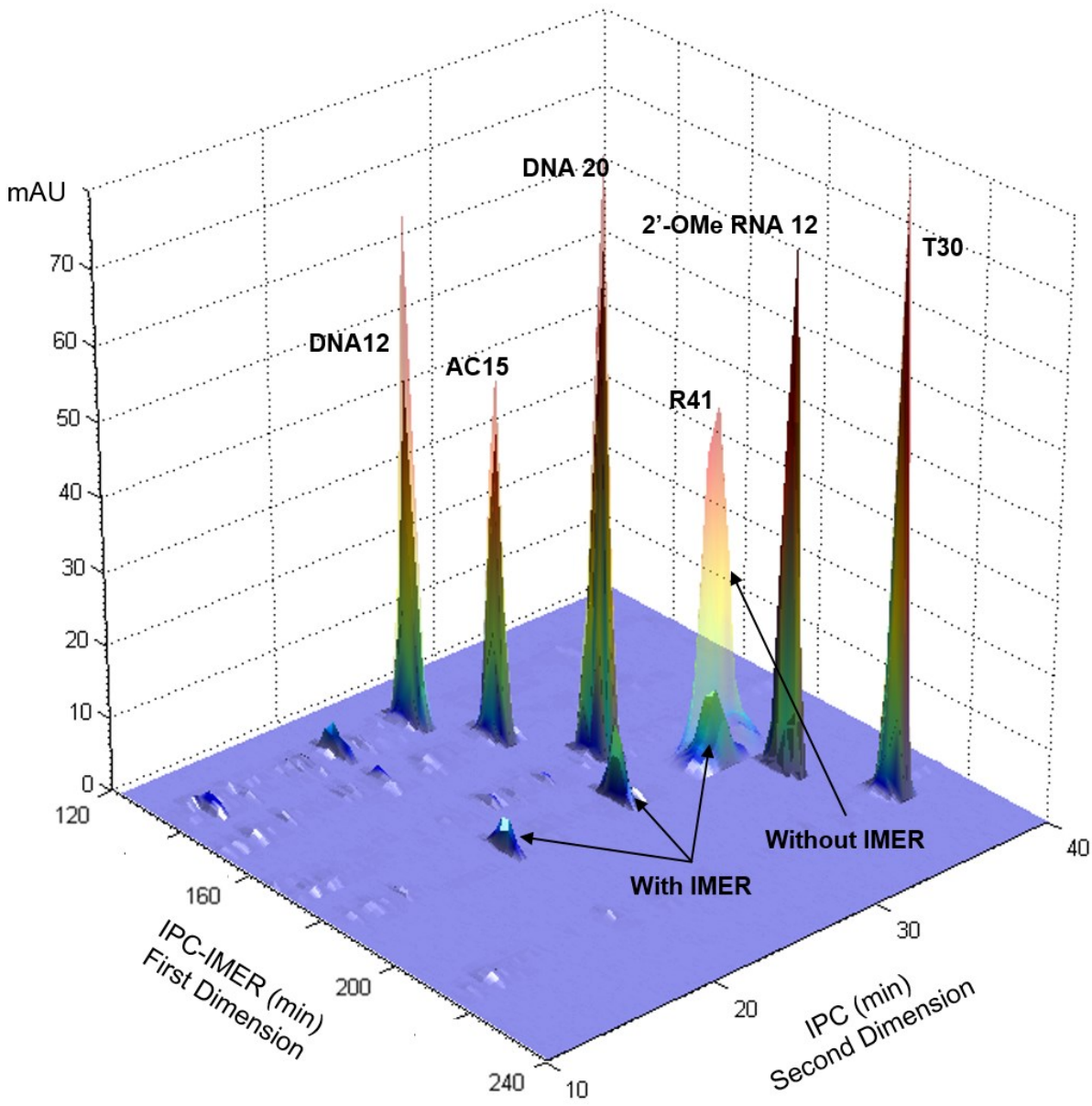


Figure 3. A) IPC chromatogram of a mixture of 6 ONs. B) IPC-IMER x IPC contour plot. C) IPC chromatogram of a fraction corresponding to the elution of the R41 ON. For conditions refer to section 2.4.



383

384

385 **Figure 4.** 3D plot overlay of an IPC-IMER x IPC and IPC-(blank IMER) x IPC analysis of a mixture of 6 ONs. For conditions refer to
386 section 2.4.

387

388

389